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Chemical profiles and angiotensin-converting enzyme inhibitory activity of *Peperomia pellucida* (L.) Kunth herb extract

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Abstract. *Peperomia pellucida* (L.) Kunth is traditionally used as a medicinal herb for hypertension. This study aimed to evaluate the chemical profile and angiotensin-converting enzyme (ACE) inhibitory activity of P. pellucida herb extract. The extract was prepared using gradual maceration and subsequently assessed for total phenolic and flavonoid contents, sensory and physicochemical characteristics, and metabolite profiling using UPLC-QToF-MS. The extract yielded 10.4% (w/w), with total phenolic and flavonoid contents of 4.33% gallic acid equivalent and 2.53% quercetin equivalent, respectively. Physicochemical analysis showed characteristic parameters within acceptable ranges, while metabolite profiling revealed several well-resolved peaks (Rt 3.5-8.0 min) corresponding to flavonoid derivatives. The extract exhibited ACE inhibitory activity with an IC_{50} value of 17.31 µg/mL. These findings provide preliminary standardization data and support the potential of *P. pellucida* extract as a promising natural source of ACE inhibitors for antihypertensive applications.

1. Introduction

The use of current control techniques as appropriate standards to ensure the quality of herbal plants as a raw material of herbal medicine products has been emphasized in the resolutions of the World Health Assembly. The control techniques of natural products as raw materials are categorized according to microscopic and macroscopic, sensory or organoleptic characteristics, total content or a specific characteristic, and physicochemical or non-specific characteristics. Other than that, metabolite profiling is critical in natural product study that giving precise data on complex chemical constituents of sample extracts from natural products [1,2].

Research on drug discovery of natural products as an antihypertensive, mainly as an angiotensin-converting enzyme (ACE) inhibitor, has continued to rise until the present. The

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application of the ACE inhibitory activity assay becomes an effective method of drug discovery based on substrate usage [3,4,5]. 3-hydroxybutyric-glycyl-glycylglycine (3HB-GGG) is one of the substrates used in the in vitro ACE inhibitor Activity assay of the natural product [6]. The action mechanism of this test (known as the Lam method) has been described in some literature [3,6,7]. In addition, this test is easy to use compared with the other substrates.

Peperomia pellucida (L) Kunth herb is traditionally used as a medicinal plant, mainly as an antihypertension. This herb is considered weeds by farmers primarily in palm oil plantations and has no economic value. Until now, *P. pellucida* has not been utilized as a commercial herbal medicine due to low yields. Moreover, the plant is one of Indonesia's most potent plants as angiotensin-converting enzyme (ACE) inhibitors, based on the study reported by Saputri et al. (2015) [8] and Kurniawan et al. (2016) [9]. Also, this herb contains phenolic, flavonoid, terpenes, alkaloids, and essential oil [10]. Phenolic or flavonoids constituent has been studied and proven to have an activity of ACE inhibitory [11,12]. However, a study on chemical profiles (including yield value, total phenol and flavonoid content, sensory and physico-chemical parameters, and metabolite profiling) that contains abundant phenolic and flavonoid content from active extract with ACE inhibitors has not been reported. Based on the best of our knowledge, the wild herb obtained from various regions throughout Indonesia has different features.

However, despite several reports on the ACE inhibitory potential of *P. pellucida*, comprehensive data integrating chemical profiles, including phenolic/flavonoid contents, physicochemical parameters, and metabolite fingerprints, with bioactivity evaluation are still limited. Therefore, this study provides preliminary standardization data and ACE inhibitory activity of *P. pellucida* extract to support its development as a potential antihypertensive herbal material.

2. Materials and Methods

2.1 Herb Materials

The wild herb *P. pellucida* was collected from a palm oil plantation in Balanti Village, Baras Sub-district, North Mamuju District, West Sulawesi Province, Indonesia. Botanical identification and authentication were conducted at the Herbarium Bogoriense, Bogor Botanical Garden, Bogor, West Java, Indonesia. A voucher specimen was deposited at the Pharmacognosy-Phytochemistry Laboratory, Faculty of Pharmacy, Universitas Indonesia, Depok, West Java, Indonesia. The plant material was air-dried, cut into small fragments, and stored at ambient temperature before extraction.

2.2 Chemical Materials and Equipment

2.2 Chemical Materials and Equipment

The chemicals utilized in this study included n-hexane, ethyl acetate, dichloromethane, methanol, acetone, and chloroform, which were procured from PT SmartLab Indonesia, Indonesia. Sodium chloride, hydrochloric acid, sodium carbonate, sulfuric acid, potassium dihydrogen phosphate, aluminium chloride, Folin-Ciocalteu reagent, glacial acetic acid, hexamethylenetetramine, acetonitrile (LiChrosolv ® for HPLC), and ethanol (Chromosolv ® for HPLC) were obtained from PT Merck, Germany, through PT Elo Kasra Utama. Captopril was supplied by PT Kimia Farma, Indonesia, while the Angiotensin-Converting Enzyme (ACE) kit WST-1 was purchased from Dojindo, Japan. The instrument employed in this study included an

analytical balance (Scout Pro), rotary vacuum evaporator (Büchi), UV-Vis spectrophotometer (Hitachi), microplate reader (Thermo Scientific), furnace oven (Memmert), and a UOLC-QToF-MS/MS system (Waters, USA).

2.3 Extraction Process

The extraction was carried out using a conventional maceration technique based on previously reported methods [13, 14], with slight modifications. Briefly, the dried powdered *P. pellucida* was macerated in n-hexane for 24 hours. After filtration, the remaining plant residue was re-macerated with methanol under identical conditions. The resulting extracts were filtered and concentrated by evaporation to yield dried extracts, which were subsequently stored at room temperature until further analysis.

2.4 Chemical Profiles Evaluation of P. pellucida Extracts

2.4.1 Determination of Total Phenols and Flavonoid Content

The total phenolic content (TPC) and total flavonoid content (TFC) of *P. Pellucida* herb extract were determined using the Folin-Ciocalteu and aluminium chloride colorimetric methods, respectively, following previously reported procedures [15-17] with minor adjustments. For TPC analysis, 0.25 mL of extract solution (100 ppm) was mixed with 3.75 mL of deionized water, followed by the addition of 0.25 mL Folin-Ciocalteu reagent. The mixture was homogenized and allowed to react in the dark for 3 minutes. Subsequently, 1.25 mL of 20% sodium bicarbonate solution was added, and the mixture was incubated at 40°C for 40 minutes. The absorbance was measured at 750 nm using a UV-Vis spectrophotometer. Gallic acid (5, 15, 30, 50, and 100 ppm) was used to construct the calibration curve. For TFC determination, 5 mL of methanolic extract solution (100 ppm) was mixed with 2 mL of 10% (w/v) aluminium chloride and 0.1 mL of 0.1 mM sodium acetate. The reaction mixture was allowed to stand for 30 minutes at room temperature. Absorbance was measured by spectrophotometry at 415 nm, and quercetin (0.5, 1, 5, 10, and 15 ppm). Each treatment was performed in three replications.

2.4.2 Determination of Sensory Parameters

Sensory or organoleptic characteristics of extract samples were determined using some parameters include odor, taste, color, and pH, which were studied as per WHO guidelines according to studies that have been previously reported [18,19].

2.4.3 Determination of Physicochemical Parameters

The physicochemical characteristic of the sample extract was conducted for the quantitative determination of non-specific parameters like water content, loss on drying, total ash content, insoluble acid content, alcohol-soluble content, water-soluble extractive, and the residual methanol content, as per WHO guidelines based on the method reported by some previous studies [20-22].

2.4.4 Metabolite Profiling Analysis

Metabolite profiling analysis was performed using a UPLC-QToF-MS/MS system based on the literature [23-25], with minor modifications to accommodate instrument conditions. Briefly, 10 mg of the extract was accurately weighed and dissolved in 100mL of LC-MS grade methanol. An aliquot of 1mL of the prepared solution was transferred into an Eppendorf tube and centrifuged at 13,000 rpm for 10 minutes. The resulting supernatant was carefully collected and transferred to a clean Eppendorf tube for analysis. Chromatographic separation was carried out using ultra-performance liquid chromatography coupled with mass spectrometry (UPLS-MS), equipped with an electrospray ionization (ESI) source and a quadrupole time of flight (QToF) mass analyzer. The injection volume was 5 μL, with a total run time of 10 minutes at a flow rate of 0.3 mL/min.

Analysis was conducted in positive ionization mode using a C18 column. The mobile phase of a gradient system of 0.1% formic acid in water and 0.1% formic acid in acetonitrile.

2.5 *Angiotensin-Converting Enzyme (ACE) Inhibition Assay*.

The angiotensin-converting enzyme (ACE) inhibitory activity was evaluated using the ACE kit=WST (Dojindi Laboratories, Japan) in accordance with the manufacturer's instructions and previously reported methods [6, 7, 26]. Catopril was used as a positive control. Briefly, 20 µL of extract solution or captopril standard (at each concentration) was added to the designated sample wells, while 20 µL was also dispensed into Blank 1 and Blank 2 wells. Subsequently, 20 µL of substrate buffer was added to all wells. Deionized water (20 µL) was added to Blank 2 wells, whereas 20 µL of enzyme working solution was added to each sample well and Blank 1 well using a multichannel pipette to minimize time variation between wells. The plate was incubated at 37°C for 1 hour. Following incubation, 200 µL of indicator working solution was added to each well, and the plate was further incubated at room temperature for 10 minutes. Absorbance was measured at 450 nm using a microplate reader. ACE inhibitory activity was calculated using the following equation:

$$\text{ACE inhibitory activity (inhibitory rate \%)} = [(A_{\text{blank 1}} - A_{\text{sample}}) / (A_{\text{blank1}} - A_{\text{blank 2}}) \text{ X } 100$$

The determination of IC_{50} was performed using the obtained absorbance from each concentration of the sample and the captopril solution. A linear regression line equation ($y = ax + b$) was made with a slope of 1.0 for the test solution and used to determine the IC_{50} of extract and captopril, respectively.

3. Results and Discussion

3.1 Extraction Process and Chemical Profiles

P. pellucida herb is a shrub containing rich in chlorophyll, which is one of the factors in extracting the secondary metabolite from the sample. Extraction using n-hexane (nonpolar solvent) aims to attract chlorophyll and lipids contained in the sample plant, and then is followed by using methanol to attract the secondary metabolite target. The organic solvent of methanol was used to extract secondary metabolites from the matrix sample. Phenols and flavonoids are a group of thermolabile compounds, so extraction by maceration is expected not to damage the phenol and flavonoid content. The liquid extract was collected and then evaporated to obtain a concentrated extract. Evaporation was performed with a rotary vacuum evaporator and a low-temperature vacuum oven.

In the present study, the methanolic extract of *P. pellucida* contains 4.33% gallic acid equivalent and 2.53% quercetin equivalent. Different growth sites, sampling times, and extraction methods can produce different total content of secondary metabolites (mainly phenols and flavonoids). Sensory or organoleptic and physicochemical parameters had specific characteristics of the sample extract (shown in **Table 1**).

Table 1. The results of pharmacognostical evaluation of *P. pellucida* herb extract

No.	Types of Parameters	Results
	Total content parameters	
1	Total phenolic content	4.33% gallic acid equivalent
2	Total flavonoid content	2.53% quercetin equivalent
	Sensory or organoleptic parameters	
3	Odor	Specific odor
4	Taste	distinctive taste
5	Color	greenish brown
6	Consistency	Semisolid mass
7	pH	6.88
	Physicochemical parameters	
8	Water content	3.62%
9	Loss on drying	4.26%
10	Total ash content	1.68%
11	Acid-insoluble content	0.05%
12	Alcohol-soluble extractive	82.46%
13	Water-soluble extractive	16.74%
14	Methanol residual content	3.9%

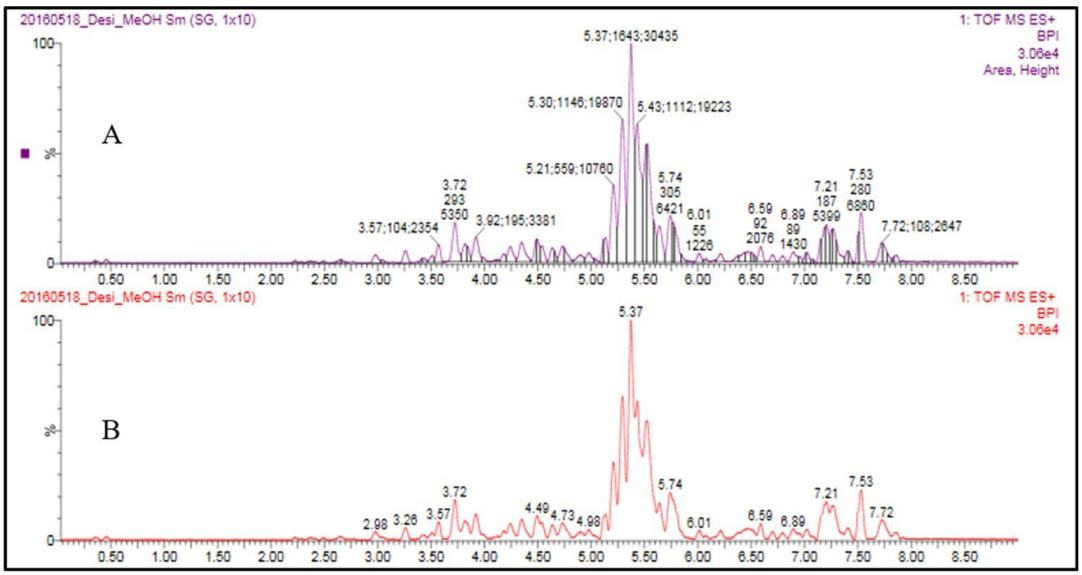


Figure 1. Peak data of metabolite profiling analysis from P. pellucida herb extract using a UPLC-QToF-MS/MS system. Where A is the total ion chromatogram (TIC) with peak smoothing and peak area integration; B is the total ion chromatogram with smoothing

Table 2. Comparison of m/z calculation based on the spectrum of *P. pellucida* herbs extract with molecular weight active compound from literature.

No	m/z value of spectrum from each Retention time value	Compound with molecular weight from references		
		Molecular Weight	Compound Name	Reference
1.	623.2878	624.59	Acteoside	[11]
2.	537.2675	538.464	Amentoflavone	[32]
3.	933.5718	934.633	Arjunin	[32]
4.	433.2751	434.353	Guaijaverin	[11]
5.	623.2878	624.54	Isorhamnetin 3-O-b-D-rutinoside	[33]
6.	477.2599/477.2639/ 477.2684/477.2691/ 477.2729/477.2755	478.406	Mearnsitrin	[32]
7.	591.2705	592.553	Proantosianidin	[29]
8.	433.2751	434.397	Prurine	
9.	433.2751	434.35	Quercetin 3-arabinopyranoside	[11]
10.	477.2599/477.2639/ 477.2684/477.2691/ 477.2729/477.2755	478.36	Quercetin glucuronide	[11]
11.	329.1459	330.292	Rhamnazin	[32]
12.	609.2759	610.521	Rutin	[33]
13.	593.2784	594.522	Apigenin	[34]

Metabolite profiling analysis was performed using UPLC-MS to predict phenolic and flavonoid compounds contained in the methanolic extract of *P. pellucida*. **Figure 1** and **Table 2** demonstrate peak data and the m/z spectrum of metabolite profiling from the methanolic extract of *P. pellucida* herbs. Based on the results of metabolite profiling analysis, using the optimun condition of UPLC-MS showed peaks with a depth and a well-separated Rt (retention time) value with a range from 3.0 to 8.0 minutes (in **Figure 1**). While based on the m/z value of the UPLC-MS spectrum demonstrated the absence of the compound for each peak of Rt value based on molecular weight data compared with references (summarized in **Table 2**). Quercetin glucuronide and mearnsitrin had the same molecular weight 478.36 at the UPLC-MS spectrum with m/z values of 477.2599, 477.2639, 477.2684, 477.2691, 477.2729, and 2755. Rutin (610.521), guaijaverin or prurin (434.397), proanthocyanin (592.553), apigenin or vicenin 2 (594.522), acteoside (624.59), isorhamnetin-3-O-b-D-rutinoside (624.54), and arjunin were probably identified at m/z value of 609.2759, 433.2751, 591.2705, 593.2784, 623.2755, 623.2759, and 933.5718, respectively while amentoflavone was predicted at m/z value of 537.2631, 537.2664, 537.2675, and 537.2772, although there is still the possibility of other compounds with the same molecular weight.

Some studies have reported that flavonoid compounds have ACE inhibitory activity, such as astragalin, kaemferol-3-O-glucoside, isoquercitrin, quercetin-3-O-glucoside [27,28], vitexin, isovitexin orientin, isoorientin, procyanidin C1 [29], luteolin, routine, rhoifolin, apigenin [28]. Hansen (1996) reported that quercetin-3-O-ramnoside and kaemferol-3-O-ramnoside work synergistically as ACE inhibitors [27]. Whereas Lacaille-Dubois (2001) concluded that flavones and procyanidins work synergistically as ACE inhibitors, whereas the isolated compound has activity compared with extracts [29].

3.2 Angiotensin-Converting Enzyme (ACE) Inhibitory Assay

ACE inhibitory activity assay was performed using the ACE kit-WST1, Dojindo, Japan, and 3-hydroxybutyric-glycyl-glycyl-glycine(3HG-GGG) as a substrate. In this assay (**Table 3**), a blank is required for method validation. A positive blank was used as a comparison of the amount of 3HB produced by ACE, while a negative blank was the absence of the ACE enzyme as a catalyst for substrate reaction. Captopril is a clinically first-choice drug as an antihypertensive with ACE-inhibiting activity. So that captopril was used as a comparison of ACE inhibitory activity against the extracted sample (100 ppm). This data was used to determine the ACE inhibitory activity (IC_{50}) value of the extract. The results of the ACE inhibitory activity of the sample extract compared with the positive control (captopril) are shown in **Table 4**.

Table 3. Test results of percent inhibition value of the extract (100 ppm).

Absorbance			Percent Inhibition value (%)
Extract sample	**Positive Blank**	**Negative Blank**	
0.4162	1.3571	0.0659	
0.4161	1.3571	0.0659	72.8702
0.4163	1.3571	0.0659	

Table 4. Results of ACE inhibitory activity (IC_{50}) by positive control (Captopril) and extract sample.

Sample	Concentration (µg/mL)	Inhibitory activity (%)	Linear Regression Formula	IC_{50} (µg/mL)
P. pellucida Extract	6.25	47.32	Y=0.2757X+45.227 R^2=0.9983	17.312
	12.5	48.90		
	25	51.39		
	100	72.93		
Captopril	$5x10^{-13}$	45.81	Y=10.692X+177.79 R^2=0.9968	$1.12x10^{-12}$
	$1x10^{-11}$	61.40		
	$5x10^{-11}$	67.10		
	$5x10^{-9}$	87.76		
	$1x10^{-8}$	93.35		

ACE inhibitory activity in vitro assay was conducted using a 3-hydroxybutyric-glycyl-glycyl-glycine substrate. The use of a substrate is a rapid method compared with other substrates. This substrate was developed and published by Lam *et al*. (2007) [6], and the reaction mechanism has been explained by literature [3,7,30]. In this study, the ACE inhibitory activity of the methanolic extract and the control positive (captopril) was measured with IC_{50} of 17.312 µg/ml and $1.12x10^{-}$

^{12} µg/ml, respectively. Based on the positive control, the obtained IC_{50} data (1.12x10-12 µg/ml) give valid results, as a study has reported by Ibadallah *et al*. (2015) [31]. According to the result, the extract of *P. pellucida* herb with chemical profiles had potential activity as an ACE inhibitor.

4. Conclusion

This study provides a comprehensive preliminary characterization of *Peperomia pellucida* herb extract by integrating chemical profiling with its angiotensin-converting enzyme (ACE) inhibitory activity. The extract demonstrated distinct sensory and physicochemical characteristics, measurable levels of phenolic and flavonoid content, and a reproducible metabolite fingerprint dominated by flavonoid derivatives. These chemical features were consistent with the extract's ACE inhibitory activity, which showed an IC_{50} value of 17.31 µg/mL. Overall, the findings offer essential baseline data for the standardization of *P. pellucida* extract as a potential antihypertensive herbal raw material. Future studies focusing on compound isolation, mechanism elucidation, and extract optimization are warranted to further support its development for pharmaceutical or nutraceutical applications.

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